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DRUG TARGET IN CANCER THERAPY

Field of the invention

5 The present invention relates to a novel drug target in cancer therapy. More closely it relates to use of the cytochrome P450 enzyme CYP2W1 and its promoter as a drug target for cancer therapy. It also relates to screening methods for obtaining therapeutic agents for cancer therapy and to therapeutic agents comprising moieties showing binding affinity for CYP2W1 and moieties with cytotoxic/anti-cancer effect.

10 Background of the invention

The cytochromes P450 are responsible for the metabolism of endogenous as well as exogenous compounds. In human at present about 80 different P450 genes have been identified of which 55 are functional whereas 25 are pseudogenes. The human P450 forms can be divided into three major groups: i) those in CYP families 5-51 of endogenous importance having usually high affinity for the substrates and being relatively well conserved during evolution, ii) those in CYP families 1-3, with usually less affinity for their substrates, being less conserved evolutionary and which exhibit important genetic polymorphisms and iii) those in family 4 with properties in between the two other groups (see Ingelman-Sundberg (2002) for a review). CYP enzymes in families 1-3 are active in metabolism of exogenous compounds and currently 19 genes encoding active enzymes have been described. The cytochromes P450 in these families are responsible for 70-80 % of all phase I dependent metabolism of clinically used drugs and participate in the metabolism of a huge number of other xenobiotic chemicals. This can lead to the metabolic activation of procarcinogens and drugs, which might exert toxic or carcinogenic effects. This capacity to activate prodrugs to cytotoxic products are seen e.g. in the metabolic activation of acetaminophen which is metabolised by CYP2E1 and CYP3A4 to a reactive imine intermediate that can cause liver hepatotoxicity. Other examples of cytochrome P450 dependent metabolic activation come from prodrugs in cancer therapy where P450 enzymes like CYP2B6 inserted by viral transfection into tumours facilitate tumour suppression upon treatment with cancer drugs

agents like e.g cyclophosphamide or ifosfamide (Waxman et al., 1999, Jounaidi and Waxman, 2001).

Summary of the invention

The invention is based on the specific expression of the cytochrome P450 enzyme CYP2W1 in 5 transformed tissue. This tissue specific expression found by the inventors can be used to target drugs and prodrugs to the transformed cells.

The invention thus relates to a compound comprising one part conferring cytotoxic and/or anti-cancer effects to the compound and one part conferring binding affinity towards CYP2W1 to the compound. It also relates to a pharmaceutical composition comprising such a compound.

10 In a further aspect the invention relates to use of CYP2W1 as a drug target in both screening for therapeutic agents and in cancer therapy.

In yet a further aspect the invention relates to the CYP2W1-promoter and use thereof in screening for therapeutic agents and in gene therapy.

15 The invention can for example be used for, but is not restricted to, the treatment of lung tumours, colon tumours or ovarian tumours, or all or any tumour expressing CYP2W1 constitutively or after administration of an inducing agent.

Brief description of the figures

20 **Figure 1**

Gene structure (A), nucleotide and predicted amino acid sequence (B) of CYP2W1. (A) Exons are shown as boxes with size in base pairs. The size of the introns is shown in kilo base pairs. (B) The stop codon is indicated by an asterisk and the proline-rich region, PPGP, and the conserved cysteine are shown in italic bold. The 3'-flanking region was obtained from the Celera Transcript 25 hCT1786066.

Figure 2

Unrooted phylogenetic tree of CYP2W1 and other human P450s belonging to family 1, 2 and 3. The scale bar for distance measurements represents 0.1 amino acid substitutions per site.

Figure 3

Real-time PCR based quantification of CYP2W1 mRNA expression in Human Tumour Multiple Tissue cDNA (MTC) Panel using gene specific primers.

Figure 4

5 Distribution of CYP2W1 mRNA in different human tissues and cell lines. (A) Multiple Tissue Expression (MTE) Array containing 42-500 ng of normalized human poly(A)⁺ RNA per dot. (B) Multiple Tissue Northern (MTN) blot containing approximately 1 µg poly(A)⁺ RNA per lane and Northern blot containing 20 µg total RNA from human liver, HepG2, HeLa, B16A2 or HEK293 cells per lane. All blots were hybridized with a ³²P-labeled probe corresponding to nucleotides 10 329-986 and exposed for 48 hours. The size markers used for the northern blot were the rRNA 28S (4.6-5.3 kb) and 18S (1.8-2.0 kb) band.

Figure 5

15 Western blot containing four lanes with HepG2 total cell extract corresponding to 40 µg protein per lane. The first two lanes were incubated with CYP2W1 antiserum, while the last two were incubated with antiserum blocked with peptide. The blot was exposed for 3 minutes.

Figure 6

CO-spectral analysis of the microsomal fraction of the pCMV4-2W1 II6 construct.

Figure 7

20 Western blot analyses of microsomal, mitochondrial and cytosolic fractions obtained from transfected HEK293 cells. Fractions corresponding to 40 µg protein were loaded per lane. The last two lanes contain human liver microsomes and cytosol corresponding to 40 µg protein. The membrane was incubated with CYP2W1 antiserum and exposed for two minutes.

Figure 8

5' flanking region deletion constructs in pGL3-Basic.

Figure 9

Comparison of luciferase activity of different promoter lengths. Luciferase assay was performed 24 hours after transfection. Basal luciferase reading (pGL3-Basic) was set at 1.0.

Figure 10

5 Shorter constructs made from genomic DNA. The region between the transcription initiation site and 78 bp upstream gave a significant increase in luciferase activity.

Figure 11

The region between the transcription initiation site and 78 bp upstream of it, which contains the TATA box, gives a significant increase in luciferase activity.

10

Detailed description of the invention

The approach for drug targeting in cancer therapy according to the invention is to utilize a specific form of cytochrome P450 only present in the transformed tissue being able to activate prodrugs converted to cytotoxic products. The present inventors have identified a novel human 15 cytochrome P450 form, CYP2W1, mainly expressed in tumour cells. This finding represents a major step of importance for the development of new potential agents effective in cancer therapy. Thus, in a first aspect the present invention relates to the use of the cytochrome P450 enzyme CYP2W1 and genetic variants thereof as a drug target in cancer therapy. CYP2W1 was found by the inventors to be selectively expressed in tumour tissues such as lung carcinoma, colon 20 carcinoma and ovarian carcinoma. A common problem in cancer therapy is the unselective toxicity exerted by cytotoxic drugs against cancer cells and their healthy counterparts. The present invention shows the potential to improve drug targeting to tumour cells solely by administering substances that can be metabolically activated by CYP2W1 to cytotoxic agents. Accordingly, the invention provides a method for screening for such substances, substrates for 25 CYP2W1, which can be modified by the enzyme to obtain a cytotoxic metabolite. This method comprises the steps:

1. Bringing a candidate prodrug into contact with CYP2W1 or a cell expressing CYP2W1.
2. Allowing CYP2W1 to act on the candidate prodrug to produce a candidate drug.

3. Analysing the candidate drug for cytotoxic/anti-cancer properties.

The candidate prodrug is preferably a non-toxic compound suitable for conventional pharmaceutical administration.

In another preferred method of screening for cytotoxic agents, anticancer agents, or other substrates, activated by CYP2W1, the enzyme is expressed using for example yeast as expression system. Other expression systems like bacterial and mammalian expression system could also be used as enzyme source. The obtained microsomes are incubated with a fluorescent substrate that is metabolised by CYP2W1. Inhibition of that reaction is then used for drug screening with different substrates. Candidate substrates to be tested, but not restricted to, could be drugs with moieties that are known to be metabolised by other members of the CYP2 family. The substrate that shows the best inhibition can be modified in order to obtain a cytotoxic metabolite. Thus the invention relates to a screening assay using CYP2W1 as a drug target.

Another aspect of the invention relates to targeting of drugs and prodrugs to cancer cells, preferably to lung, colon or ovarian tumour cells. Since cytochromes P450 are partially expressed on the cell surface, drugs and/or prodrugs can also be targeted to cancer cells by the coupling of them to ligands which bind specifically to CYP2W1. In this aspect, "prodrug" means a substance which will be converted to a cytotoxic and/or anti-cancer drug upon binding to a cell expressing CYP2W1 on its surface. The drug to be administered in this way could be any anticancer/cytotoxic drug known in the art. The prodrug could be a prodrug identified by the screening method described above or any other compound which is activated to a cytotoxic or anti-cancer agent by CYP2W1. The ligand to which the drug/prodrug should be coupled could be any molecule that binds specifically to CYP2W1 bound on the cell surface, i.e. does not show any substantial specific binding affinity towards any other endogenous or exogenous substances. The ligand could be an antibody, preferably a monoclonal antibody or a binding fragment thereof, which can be prepared by standard techniques. Pharmaceutical compositions comprising the drug/prodrug-ligand compound could be prepared by techniques known in the art.

Yet another aspect of the invention relates to the combinatorial use of a prodrug to be metabolised, and a substance that can induce the expression of CYP2W1 in the tumour cells, thus resulting in higher concentrations of the cytotoxic metabolite at the site of the tumour .

In a further aspect, the invention relates to a method of providing therapeutic agents for cancer therapy, comprising screening for such agents by using CYP2W1 as a drug target.

5 The therapeutic agents shall modulate the activity of CYP2W1, such as induce or increase the activity thereof. This method comprises the steps:

1. Bringing a candidate agent into contact with a cell capable of expressing CYP2W1 or a reporter gene operably linked to the regulatory sequences of CYP2W1.
2. Measuring changes in expression and/or activity of CYP2W1

In order to screen for agents inducing the expression of CYP2W1 different cell lines can be used.

10 One example of possible cell line is the human hepatoma cell line HepG2, which is constitutively expressing CYP2W1. The agent to be tested will be administered to the supplement medium and the induction of the CYP2W1 expression can be tested on the RNA, protein and/or enzymatic activity levels. Candidate agents to be tested, but not restricted to, could be known inducers of cytochrome P450s belonging to the CYP2 and CYP3 family, including those being efficient substrates for the nuclear receptors PXR, LXR, FXR and CAR.

15 In a yet further aspect, the invention relates to a method of treating cancer, comprising giving a patient in need thereof a therapeutically effective amount of a substance activated by the enzyme CYP2W1 and/or inducing the enzyme CYP2W1.

20 For example, the substance may be activated to an anticancer agent or a cytotoxic substance.

The invention also relates to the use of the CYP2W1 promoter (SEQ ID NO: 10) in gene therapy. This aspect includes the use of the promoter in the manufacture of a medicament, preferably a medicament for use in cancer therapy, even more preferably for use in treatment of lung tumours, 25 colon tumours or ovarian tumours. This aspect also includes the use of DNA-molecules containing the CYP2W1 promoter in pharmaceutical compositions, such DNA-molecules could be, for example, vectors for delivery of the promoter to the patient in need thereof.

30 In an even further aspect the invention relates to the use of the CYP2W1 promoter in screening for modulators, such as inducers or enhancers, of CYP2W1. The promoter can be linked to a variety of reporter systems known in the art in order to study the effects of candidate modulators.

Experimental

The present invention will be described in more detail below in an experimental section.

Materials and Methods

5 Bioinformatics

The BLASTN search algorithm was used to search the Celera sequence databases (<http://www.celera.com>) for sequences related to the previously reported CYP2W1 partial cDNA sequence (GenBank Accession No. AK000366). The amino acid sequence of CYP2W1 was compared with the amino acid sequences of other human P450s using multiple sequence 10 alignment. An unrooted phylogenetic tree was calculated using ClustalW 1.8 (Thompson et al., 1997) and visualized using the program unrooted (<http://pbil.univ-lyon1.fr/software/unrooted.html>).

RNA Isolation and 1st strand cDNA Synthesis

Total RNA was isolated from cultured HepG2 and B16A2 cells using the TRIZOLE Reagent 15 (Invitrogen, Rockville, MD) based on the acid guanidine thiocyanate phenol chloroform extraction method, whereas total RNA from HEK293 and HeLa cells was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) Reverse transcriptase was performed using MMLV Reverse Transcriptase (Invitrogen, Rockville, MD) according to the manufacturer's instructions.

Amplification and Sequencing

20 cDNA from HepG2 cells was used as a template in PCR reactions with CYP2W1 specific primers. The enzyme used for amplification was Taq DNA Polymerase (ABgene, Epsom, UK). The PCR products generated were sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA), according to the manufacturers instructions, and analyzed on an ABI PRISM 377 DNA Sequencer (Applied 25 Biosystems, Foster City, CA).

Multiple Tissue cDNA Panels

cDNA from Human Multiple Tissue cDNA (MTC) Panel I and II, Human Digestive System MTC Panel, Human Fetal MTC Panel and Human Tumour MTC Panel (Clontech, Palo Alto,

CA) were used as templates in a PCR-reaction using the CYP2W1 specific primer pair, 5'-GAGGTGGAGGCATCTTCTCATCTG-3'/5'-CTGGATCAGGGCGTCCACATAGCTG-3'. A reaction without cDNA was run as a negative control in each experiment. The PCR amplification was performed in the presence of 1 mM MgCl₂, 0.2 mM of each dNTP, 0.4 µM of each primer, 0.625 U Taq DNA Polymerase (ABgene, Epsom, UK), 1x Reaction Buffer IV (75 mM Tris-HCl pH 8.8 at 25°C, 20 mM (NH₄)₂SO₄, 0.01% (v/v) Tween 20) and 0.5 ng cDNA. The PCR reaction was performed by an initial denaturation step at 94°C for 3 min, after which the amplification was carried out for the indicated number of cycles, with denaturation at 94°C for 45s, annealing at 60°C for 30s, and extension at 72°C for 1 min and 30s, and a final extension step of 72°C for 10 min. HepG2 cDNA was used as a positive control, and the quality of the cDNA in the MTC panels was ensured by using the G3PDH control primers, supplied by the manufacturer. Amplification was performed under the same conditions as described above. The PCR products were separated and detected on a 1.2% ethidium-bromide stained agarose gel. The PCR product generated was sequenced and analyzed, as described above, in order to ensure specificity of the reaction.

Real-time quantitative PCR detection

CYP2W1 mRNA expression was quantified in Human Tumour MTC Panel using the Smart Cycler® System (Cepheid, Sunnyvale, CA). The quantitative real-time PCR assay was carried out using the CYP2W1 gene specific primer pair, 5'-AGCTATGTGGACGCCCTGATCCA-3'/5'-ACGCGGTCTAGCTCCTCCTGCAC-3', and SYBR®Green PCR Master Mix (Applied Biosystems, Foster City, CA). The quantification was performed using 0.5 ng of each cDNA sample. To avoid detection of any possible contaminating genomic DNA the primer pair was designed so the resulting product spans exon junctions. Standard curves were constructed with the use of serial 10-fold dilutions ranging from 10⁻² fmol/µl to 10⁻⁹ fmol/µl of an accurately determined concentration of a CYP2W1 full-length cDNA fragment. The data was analyzed using the Smart Cycler® Software (Cepheid, Sunnyvale, CA).

Analysis of mRNA by Northern and Dot Blot

A Human Multiple Tissue Expression (MTE) Array (Clontech, Palo Alto, CA) and a Multiple Tissue Northern (MTN) Blot (Clontech, Palo Alto, CA) were hybridized with a ³²P-labeled

CYP2W1 probe, corresponding to nucleotides 329-986, which was generated by PCR and labeled using the Radprime DNA labelling system (Invitrogen, Rockville, MD). Hybridization was performed according to the manufacturer's instructions.

The ³²P-labeled CYP2W1 probe was also hybridized to total RNA from HepG2, B16A2, 5 HEK293 and HeLa cells and total RNA from human liver (provided by Anna Westlind). In particular, 20 µg RNA per lane was loaded on a 1.2% agarose/formaldehyde denaturing gel and subjected to electrophoresis using standard procedures (Struhl, 1993). The RNA was transferred to a Hybond-N+ filter (Amersham Pharmacia Biotech, Uppsala, Sweden) and hybridized using ExpressHyb Hybridization Solution (Clontech, Palo Alto, CA). Prehybridization was carried out 10 at 65°C for 30 minutes, and hybridisation was carried out at the same temperature over night.

After hybridisation all three blots were exposed to an imaging plate and analysed by a Bas-1800II phosphoimager using the Image Gauge software (Fujifilm, Samford, CT).

Cell Culture Conditions

HepG2 and HEK293 cells were cultured in Minimum Essential Medium supplemented with 10% 15 fetal bovine serum, 1mM sodium pyruvate, 1% non-essential amino acids, 100 IU/ml penicillin and 100 µg/ml streptomycin. HeLa cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 100 IU/ml penicillin and 100 µg/ml streptomycin. B16A2 cells were cultured in William's E Medium supplemented 20 with 5% fetal bovine serum, 0.6 µM hydrocortisone, 0.25 µg/ml insulin, 100 IU/ml penicillin and 100 µg/ml streptomycin. All cells were cultured in humidified 5% CO₂ at 37°C. All cell culture media and supplements were obtained from Invitrogen, Rockville, MD.

CYP2W1 Antiserum

An 15 amino acid long peptide, TMRPRAQALCAVPRP, corresponding to the C-terminus of CYP2W1 was synthesized and coupled to keyhole limpet hemocyanin (Neosystems, Strasbourg, 25 France). Immunization of two rabbits was carried out with injections at day 0, 14, 27 and 56. Bleedings were performed before the first injection to obtain pre-immune serum, and after 39, 67 and 96 days to obtain the CYP2W1-specific antiserum.

Preparation of Total Cell Extracts and Western Blot

The human cell lines HepG2, HEK293, HeLa and B16A2 were analysed for CYP2W1 expression using western blot. Cells were washed twice, scraped into ice-cold PBS and pelleted by centrifugation at 17,320x g. The pelleted cells were resuspended in a phosphate buffer, 5 containing 50 mM NaPO₄ pH 7.4, 0.1mM EDTA and 10% glycerol, and lysed by sonication. After centrifugation at 17,320x g, supernatants were collected and protein concentration was determined according to Lowry. 40 µg total cell extract as well as human liver microsomes and cytosol , were subjected to SDS-PAGE using a 10% gel. The proteins were subsequently transferred to a Hybond-C extra membrane (Amersham Pharmacia Biotech, Uppsala, Sweden). 10 After transfer, the membrane was blocked in TBS containing 0.05% (v/v) Tween 20 and 5% fat-free milk, and incubated with a 1:5000 dilution of the CYP2W1 antiserum. This was followed by an incubation with 1:2000 diluted horseradish peroxidase-conjugated anti-rabbit 15 immunoglobulins and detection using SuperSignal West Pico Chemiluminiscent Substrate (Pierce, Rockford, IL). Blocking experiments were performed using antiserum pre-incubated with peptide. The CYP2W1 antiserum was incubated together with the peptide used for immunisation, at a concentration of 3 µg peptide per µl antiserum. Incubation was performed at room 15 temperature for 2 hours under gentle agitation. After incubation, the antiserum was diluted and immunoblotting was carried out, as described above.

Construction of CYP2W1 Expression Plasmids

20 A full-length cDNA clone of CYP2W1 was amplified from cDNA derived from HepG2 cells using Elongase Enzyme Mix (Invitrogen, Rockville, MD). The primer pair used was 5'-
GACAGATCTATGGCCCTGCTGCTCTTG-3'/5'-
GACTCTAGACTAGGGCCTGGGCACCGCA-3'. Restriction sites for *Bgl*II and *Xba*I (underlined) were introduced by the primers. The PCR reaction contained 0.2 mM of each dNTP, 25 0.4 µM of each primer, 2.5 U Elongase Enzyme Mix (Clontech, Palo Alto, CA), and a Buffer A/Buffer B ratio corresponding to 1.7 mM MgCl₂ (5x Buffer A containing 300 mM Tris-SO₄ pH 9.1, 90 mM (NH₄)₂SO₄, and 5 mM MgSO₄; 5x Buffer B containing 300 mM Tris-SO₄ pH 9.1, 90 mM (NH₄)₂SO₄, and 10 mM MgSO₄). The conditions for the reaction were as follows, a denaturation step at 94°C for 1 min followed by 70°C for 1 min when Buffer A/Buffer B and 30 Elongase Enzyme Mix were added as a hot start. Before amplification an additional denaturation

step at 94°C for 1 min was performed. The amplification was carried out with denaturation at 94°C for 30s, annealing at 52°C for 30s, and extension at 68°C for 2 min for 35 cycles, and a final extension step of 68°C for 7 min. The obtained PCR product was digested with *Bg*II and *Xba*I (Invitrogen, Rockville, MD) and ligated into the expression vector pCMV4, cut with the same restriction enzymes as the PCR product. The sequence inserted was verified by DNA sequencing.

Heterologous Expression in Mammalian Cells

The HEK293 cell line was used for expression of the CYP2W1 protein. The cells were transfected with 30 µg pCMV4-2W1 plasmid per 15 cm cell culture dish using Lipofectamine 2000 (Invitrogen, Rockville, MD) according to the manufacturers instructions. Cells transfected with only pCMV4 was used as a negative control. The cells were harvested 66 h after transfection and homogenized in 10 mM Tris/HCl pH 7.4 containing 1 mM EDTA, 0.5 M sucrose and Complete protease inhibitor (Roche, Mannheim, Germany). The homogenate was fractionated by centrifugation, and the microsomal fraction was used for estimation of the total P450 content. The microsomal, mitochondrial and cytosolic fractions were subjected to western blot for detection of the CYP2W1 protein, as described above.

Identification of promoter

Varying lengths of the 5' flanking region of the *CYP2W1* gene were subcloned into the reporter vector, pGL3-Basic. These were transfected into the hepatoma cell line HepG2 (Lipofectamine 2000, Invitrogen, and Tfx-20 Reagent, Promega) 24 hours prior to luciferase assay (Dual-Luciferase Reporter Assay System, Promega). The region that gave the highest luciferase activity was further analyzed by preparing constructs of shorter intervals. A transcription factor search was the basis for making these constructs.

25

Results

A number of genomic clones related to CYP2W1 were identified and aligned together in order to obtain the *CYP2W1* gene sequence. A Celera transcript, hCT1786066, was also identified which

contained the 3' untranslated region of the cDNA. The *CYP2W1* gene was more than 5.5 kb long and showed the typical family 2 gene structure with nine exons (Fig. 1A). The obtained gene sequence was used when designing primers for the amplification and sequencing of the entire HepG2 *CYP2W1* cDNA. Sequencing resulted in an open reading frame of 1473 nucleotides, 5 which encodes a 490 amino acid long polypeptide. The nucleotide and predicted amino acid sequence of *CYP2W1* are shown in figure 1B. *CYP2W1* was found to contain some typical structural features associated with P450s, including a hydrophobic NH₂-terminal, the proline-rich region and the conserved cysteine, which is the fifth heme iron ligand. The *CYP2W1* cDNA sequence described here differs extensively from the previously reported partial sequence 10 (GenBank Accession No. AK000366) with respect to the N- and C-terminal exons.

An unrooted phylogenetic tree of *CYP2W1* and other human P450s is shown in figure 2. It shows that *CYP2W1* align together with the other family 2 members, but it does not seem to be closely related to any of them. The enzymes within family 2 with highest identity to *CYP2W1* 15 are *CYP2D6* (42%) and *CYP2S1* (40%).

The tissue-specific *CYP2W1* mRNA distribution was analysed by using MTC panels by PCR. The cDNA samples in the MTC panels were pooled from several individuals and normalized with at least four different housekeeping genes. As shown in Fig 3 and Table 1 the tissues that 20 showed the highest expression of *CYP2W1* mRNA were the tumour tissues lung carcinoma and colon adenocarcinoma. Moderate expression was seen in for example fetal lung, but the expression levels seen in the majority of normal adult and fetal tissues were low or absent. A rough calculation about the difference in expression between the tumour cells having highest *CYP2W1* mRNA and normal tissue reveals a 10,000-100,000 fold difference.

Table 1

Tissue specific expression of CYP2W1 revealed by PCR analyses using gene specific primers and different Human Multiple Tissue cDNA (MTC) Panels. The number of cycles required to obtain a visible product band on an ethidium-bromide stained gel is given in the column. The abbreviation nd stands for 5 not detected, which means that no product band could be seen after 40 cycles of amplification.

Panel	Tissue	Cycles for Detection
Human MTC Panel I	Brain	nd
	Heart	nd
	Kidney	36-40
	Liver	36-40
	Lung	nd
	Pancreas	31-35
	Placenta	36-40
	Skeletal Muscle	36-40
Human MTC Panel II	Colon	31-35
	Ovary	36-40
	Peripheral Blood Leukocytes	nd
	Prostate	31-35
	Small Intestine	nd
	Spleen	nd
	Testis	nd
	Thymus	nd
	Cecum	36-40
	Colon, Ascending	36-40
Human Digestive System MTC Panel	Colon, Descending	36-40
	Colon, Transverse	36-40
	Duodenum	31-35
	Esophagus	36-40
	Ileocecum	36-40
	Ileum	31-35
	Jejunum	26-30
	Liver	31-35
	Rectum	26-30
	Stomach	31-35

Human Fetal MTC Panel	Brain	nd
	Heart	nd
	Kidney	31-35
	Liver	31-35
	Lung	26-30
	Skeletal Muscle	nd
	Spleen	nd
	Thymus	nd
Human Tumour MTC Panel	Breast Carcinoma (GI-101)	nd
	Lung Carcinoma (LX-1)	21-25
	Colon Adenocarcinoma (CX-1)	21-25
	Lung (GI-117)	36-40
	Prostatic Adenocarcinoma (PC3)	31-35
	Colon Adenocarcinoma (GI-112)	36-40
	Ovarian Carcinoma (GI-102)	26-30
	Pancreatic Adenocarcinoma (GI-103)	nd

The mRNA distribution was also examined using different mRNA blots, like MTE Array, MTN blot and Northern blotting. The mRNA amounts on the MTE Array have been normalized using eight different housekeeping genes, and the MTN blot has been normalized with respect to β -

5 actin. Using these two methods for mRNA analysis no signals, and hence no expression, could be observed in any of the tissues (Fig. 4A and B). A Northern blot analysis was also performed using RNA from HepG2, B16A2, HEK293 and HeLa cells and human liver. In HepG2 cells a strong signal corresponding to a transcript of approximately 2.3 kb was observed (Fig.4B), which corresponds well to the sequence shown in Figure 1B. No signals could be obtained in the other 10 cell lines or in human liver, which is consistent with the low CYP2W1 mRNA expression seen in liver using the MTC panels.

A CYP2W1 antiserum was produced by immunization of rabbits, using the C-terminal sequence of CYP2W1. The cell lines examined for the presence of CYP2W1 using western blotting were 15 HepG2, HEK293, HeLa and B16A2, and in addition, we also determined the content in human liver microsomes and cytosol. The membranes containing HepG2 total cell extract and incubated with CYP2W1 antiserum recognized two bands of approximately 54 and 52 kDa as seen in

Figure 5. The upper band is the result of unspecific binding to a cytosolic protein, whereas the size of the lower band corresponds well to the calculated CYP2W1 protein sequence of 490 amino acids. When immunoblotting was made using pre-immune serum (data not shown) or antiserum blocked with the immunizing peptide the previously seen bands were abolished (Fig.

5 5). No band corresponding to CYP2W1 was seen in human liver or in the other cell lines examined (data not shown). Cross-reactivity with other P450s could be excluded since no band was observed in human liver microsomes, which normally shows high expression of most P450s in families 1-3.

10 CYP2W1 was heterologously expressed in HEK293 cells using three different pCMV4-2W1 constructs (II6, III6 and III7) and empty pCMV4 vector as a negative control. The microsomal fraction of the transfected cells was used for an estimation of the P450 content by spectral analysis of the reduced CO-bound form (Fig. 6). A peak was observed at approximately 450 nm indicating presence of active P450 enzyme. The amount of P450 obtained was 5 pmol/mg 15 microsomal protein. The microsomal, mitochondrial and cytosolic fractions were subjected to western blotting for detection of the CYP2W1 protein (Fig. 7). A strong band indicating CYP2W1 were seen in the microsomal and mitochondrial fractions transfected with all pCMV4-2W1 constructs whereas no bands could be seen in the negative control.

20 Different parts of the 5'-flanking region of the CYP2W1 gene were subcloned to a reporter vector pGL3-Basic which bears the luciferase gene. Hepatoma cells HepG2 were used for the transfection experiments. Initial analysis of different lengths of the *CYP2W1* promoter region (Figure 8) suggests that the region between 130 bp and 500 bp upstream of the transcription initiation site (Figure 9, Subject 212) may possess a binding site for a transcription factor that can 25 maximally drive the expression of CYP2W1. Shorter fragments from this region were analyzed (Figure 10). The shortest fragment included in the experiment gave a significant increase in luciferase activity (Figure 10) and this region contains the TATA box where RNA polymerase II binds and initiates transcription (Figure 11). Several transcriptional factors and activators are expected to bind to this region and may also affect the level of expression of CYP2W1.

It is concluded that the region between -1 and -130 bp, SEQ ID NO: 10, constitutes a very strong promoter for expression of CYP2W1 in the hepatoma cells.

Discussion

5 Alignment of genomic clones allowed the construction of the gene structure of CYP2W1. Based upon this it was possible to make a full length cDNA construct using mRNA from HepG2 cells which revealed a P450 enzyme with expected length and sequence motifs. Semiquantitative analysis for the amount of mRNA expressed in human MTC panels revealed the highest expression in a lung, a colon and an ovarian tumour, whereas in general only small mRNA
10 expression was seen in preparations from adult tissues. Based upon the number of cycles during PCR necessary to amplify the product a rough calculation reveals 10,000-100,000 fold higher CYP2W1 mRNA expressed in these tumour cells than in non-transformed cells. Further quantitative estimations are now done using Northern blot filters from different human tumours. The inventors detected significant mRNA expression in HepG2 cells whereas, by contrast, they
15 could not see any significant expression as determined by Northern blotting in different tissues from adult human.

The inventors successfully obtained a specific antiserum to the protein. Western blotting experiments revealed that no significant expression was seen in human liver, but that high
20 expression was seen in the transformed cell line HepG2. This is promising in order to obtain specific Western blotting analyses for detection of the enzyme in different tumours. In HEK293 cells heterologously expressed with CYP2W1 the expression of three bands was seen with slightly different molecular weights. The significance of this observation is currently evaluated.

25 Large cultivation of HEK293 cells transfected with pCMV-CYP2W1 cDNA allowed the preparation of microsomes useful for detection of spectrally identifiable CYP2W1. The results showed a typical P450 spectrum indicating proper folding of the enzyme being catalytically active.
30 The transformed cells line HepG2 has not been described to express any known form of microsomal cytochrome P450 at significant levels. Unexpectedly, the present inventors found

relatively high CYP2W1 expression as revealed by the Western blotting experiments in HepG2 cells. The background for this is unknown but current work aims at identifying the transcription factors and cis acting elements required for this expression. In addition, the semiquantitative determination of CYP2W1 in some different human tumours revealed an exceptionally high 5 expression.

The present inventors propose use of the enzyme CYP2W1 as a drug target for the development of cytotoxic drugs aimed for cancer therapy. Previously the extrahepatic CYP1B1 form has been shown to be more abundantly expressed in tumours than in non-transformed tissue whereas 10 CYP2W1 appears to represent a much more important example of tumour specific cytochrome P450 expression.

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